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Airway Epithelial cGAS Is Critical for Induction of Experimental Allergic Airway Inflammation

Yinling Han,*,[1] Lin Chen,*,[1] Huiwen Liu,* Zhangchu Jin,* Yinfang Wu,* Yanping Wu,* Wen Li,* Songmin Ying,* Zhihua Chen,* Huahao Shen,*,†,[1] and Fugui Yan*,[1]

DNA damage could lead to the accumulation of cytosolic DNA, and the cytosolic DNA–sensing pathway has been implicated in multiple inflammatory diseases. However, the role of cytosolic DNA–sensing pathway in asthma pathogenesis is still unclear. This article explored the role of airway epithelial cyclic GMP-AMP synthase (cGAS), the major sensor of cytosolic dsDNA, in asthma pathogenesis. Cytosolic dsDNA accumulation in airway epithelial cells (ECs) was detected in the setting of allergic inflammation both in vitro and in vivo. Mice with cGAS deletion in airway ECs were used for OVA- or house dust mite (HDM)–induced allergic airway inflammation. Additionally, the effects of cGAS knockdown on IL-33–induced GM-CSF production and the mechanisms by which IL-33 induced cytosolic dsDNA accumulation in human bronchial epithelial (HBE) cells were explored. Increased accumulation of cytosolic dsDNA was observed in airway epithelium of OVA- or HDM-challenged mice and in HBE cells treated with IL-33. Deletion of cGAS in the airway ECs of mice significantly attenuated the allergic airway inflammation induced by OVA or HDM. Mechanistically, cGAS participates in promoting T_H2 immunity likely via regulating the production of airway epithelial GM-CSF. Furthermore, Mito-TEMPO could reduce IL-33–induced cytoplasmic dsDNA accumulation in HBE cells possibly through suppressing the release of mitochondrial DNA into the cytosol. In conclusion, airway epithelial cGAS plays an important role via sensing the cytosolic dsDNA in asthma pathogenesis and could serve as a promising therapeutic target against allergic airway inflammation. ***The Journal of Immunology*****, 2020, 204: 1437–1447.**

Allergic asthma is a chronic inflammatory pulmonary disease associated with aberrant innate and adaptive immune responses to allergens. Airway epithelial cells (ECs) represent the first line of defense against microorganisms, gases, and allergens and can express many pattern recognition receptors (PRRs) to respond to pathogen-associated molecular patterns. The activation of epithelial PRRs leads to release of cytokines, such as GM-CSF, IL-25, and IL-33 (1). These innate cytokines can activate dendritic cells (DCs), eosinophils, mast cells, and basophils to promote T_H2 immunity. Thus, the airway epithelium is an important player in asthma pathogenesis. However, the mechanisms by which airway ECs influence immune cells in asthma remain to be elucidated.

Accumulating evidence indicates that DNA damage response triggers inflammatory response (2). DNA normally resides in the nucleus and mitochondria, whereas DNA damage results in the accumulation of cytosolic DNA, which serves as a danger-associated molecular pattern to trigger immune responses. Recent studies have demonstrated that the cytosolic DNA–sensing pathway could be the major link between DNA damage and innate immunity (3, 4). DNA damage including DNA double-strand breaks has also been found in the bronchial epithelium of asthmatic mice, implicated as an underlying driver of asthma pathophysiology (5, 6). However, heretofore, there is no research to clarify whether upregulation of cytosolic DNA is existed in asthmatic airway ECs. Moreover, whether the cytosolic DNA–sensing pathway in airway epithelium exerts any effects on asthma pathogenesis is still unclear.

Cyclic GMP-AMP synthase (cGAS) is a newly discovered sensor that detects cytosolic DNA as a danger-associated molecular pattern and induces type I IFNs and other cytokines (7). DNA binds to cGAS in a sequence-independent manner, and this binding induces a conformational change of the catalytic center of cGAS such that this enzyme can convert GTP and ATP into the second messenger cyclic GMP-AMP. This cyclic GMP-AMP molecule is an endogenous high-affinity ligand for stimulator of IFN genes (STING), which subsequently recruits and activates TANK-binding kinase 1 (TBK1) and IFN regulatory factor 3 (IRF3) via a phosphorylation-dependent mechanism. STING also activates NF-κB, which functions together with IRF3 to turn on the transcription of type I IFNs and other cytokines (8). Previous studies have shown that cGAS is critical for the antiviral innate immune response in mouse embryonic fibroblasts as well as obesity-induced inflammation and insulin resistance through sensing cytosolic mitochondrial DNA (mtDNA) (9, 10). A recent study has demonstrated that rhinovirus infection triggers dsDNA

*Key Laboratory of Respiratory Disease of Zhejiang Province, Department of Respiratory and Critical Care Medicine, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang 310009, China; and †State Key Lab of Respiratory Disease, Guangzhou, Guangdong 510120, China

[1]These authors contributed equally to this work.

ORCID: 0000-0003-3060-9805 (F.Y.).

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Address correspondence and reprint requests to Huahao Shen and Fugui Yan, Second Affiliated Hospital of Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou 310009, China. E-mail addresses: huahaoshen@zju.edu.cn (H.S.) and yfg0726@zju.edu.cn (F.Y.)

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; cGAS, cyclic GMP-AMP synthase; DC, dendritic cell; EC, epithelial cell; HBE, human bronchial epithelial; HDM, house dust mite; IRF3, IFN regulatory factor 3; mtDNA, mitochondrial DNA; NETosis, neutrophil extracellular trap formation; PAS, periodic acid–Schiff; PRR, pattern recognition receptor; siRNA, small interfering RNA; STING, stimulator of IFN genes; WT, wild-type.

release associated with neutrophil extracellular trap formation (NETosis), which promotes type 2 allergic asthma exacerbation (11). In this study, we hypothesized that various allergens and inflammatory cytokines may lead to release of cytosolic DNA of airway epithelium, and cGAS may play a key role through sensing cytosolic DNA and subsequently triggering T_H2-immune response in asthma pathogenesis.

In this study, we demonstrated increased accumulation of cytosolic dsDNA in airway ECs of OVA and house dust mite (HDM)–induced asthmatic mice. Furthermore, we found that cGAS deletion in airway ECs significantly attenuated OVA- or HDM-induced airway eosinophilic inflammation, mucus overproduction, and airway hyperresponsiveness (AHR).

Materials and Methods

Experimental animals

CC10-rtTAtg/-or (tetO)7CMV-Cretg/-transgenic mice (C57BL/6 background) were provided by Y. Ke (Zhejiang University School of Medicine). $cGAS^{flox/flox}$ mice (C57BL/6 background) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Jiangsu, China). CC10-rtTAtg/-or (tetO)7CMV-Cretg/-transgenic mice were mated with $cGAS^{flox/flox}$ mice to generate double-transgenic CC10-rtTA/$cGAS^{flox/flox}$ or (tetO)7-Cre/$cGAS^{flox/flox}$ mice. Then, these mice were mated to generate CC10-rtTA/(tetO)7-Cre/$cGAS^{flox/flox}$ triple-transgenic mice. Triple-transgenic mice and littermate control mice (CC10-rtTA/$cGAS^{flox/flox}$, [tetO]7-Cre/$cGAS^{flox/flox}$ and $cGAS^{flox/flox}$) were used for the experiments. The genomic DNA from tails or lungs was extracted for genotyping.

To induce the activation of Cre recombinase in transgenic mice, 6- to 8-wk-old mice were fed with doxycycline in their drinking water (2 mg/ml) for 20 d. After confirming the knockdown effect, the CC10-rtTA/(tetO)7-Cre/$cGAS^{flox}$/mice treated with doxycycline were then designated as $cGAS^{\triangle/\triangle}$ mice (Supplemental Fig. 1). Littermate $cGAS^{flox/flox}$ mice and Cre mice are used as the control groups in our experiments. The gender of the mice between treatment and control groups was strictly matched. C57BL/6 mice (18–20 g) were purchased from the Animal Center of Slaccas (Shanghai, China) and maintained in the animal facility of the laboratory animal center of Zhejiang University. All animal experiments were in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee at Zhejiang University.

Induction of allergic airway inflammation

Three different allergic airway inflammation protocols were obtained in our study. Mice were immunized i.p. with 100 ml of 20 mg of chicken OVA (Sigma-Aldrich, St Louis, MO) emulsified in Imject alum (2.25 mg of Al[OH]3/2 mg of Mg[OH]2; Thermo Fisher Scientific, Waltham, MA) on days 0 and 14 (OVA/alum model). On conventional model, mice were then challenged with an aerosol of 1% (OVA/alum) in saline for 40 min by means of ultrasonic nebulization (DeVilbiss, Somerset, PA) on days 24, 25, and 26. Twenty-four hours after the last OVA challenge, AHR was measured and then all mice were sacrificed for further analysis. While on short time point model, mice were challenged on day 24 as same way above and sacrificed for analysis 3, 6, and 12 h later.

For the HDM model, twice-daily (days 0 and 7) intratracheal injections of 10 mg of HDM (Greer Standardized Mite [*Dermatophagoides pteronyssinus*]) Extract dissolved in 50 μl of saline were made in pentobarbital-anesthetized mice. Then mice were challenged on day 14 in the same way. Control mice were challenged with saline alone. Twenty-four or seventy-two hours after the last challenge, mice were sacrificed for further analysis.

Bronchoalveolar lavage (BAL) fluid was collected, and the lungs were removed to 10% formalin (left lung) or kept in tissue culture medium on ice for homogenization (right lung) and subsequent processing.

For each allergic airway model, the results were repeated twice. Five to seven mice were used for each group in each model.

Measurement of AHR

Airway responsiveness was determined invasively based on lung resistance after challenge with aerosolized methacholine (Sigma-Aldrich). Mice were challenged with methacholine aerosol in increasing concentrations (0, 1.5625, 3.125, 6.25, 12.5, and 25 mg/ml in saline) after the final OVA challenge as described above by using the Buxco FinePoint (Buxco Electronics, Troy, NY). Data on lung resistance were continuously collected, and mean values were selected to express changes in airway function and regarded as one form of inflammation expression.

BAL fluid and differential cell counts

After mice were sacrificed, the BAL cells were collected by injecting 0.4 ml of ice-cold PBS into the trachea through a 22-inch i.v. catheter, which was repeated three times to gather roughly 1 ml of BAL fluid. Total numbers of cells in BAL fluid were counted with a Neubauer chamber. In addition, after cytospin, based on Wright-Giemsa staining, numbers of eosinophils, neutrophils, lymphocytes, and macrophages in a total of 200 cells were counted and classified under a microscope. Results are expressed as the number of cells per milliliter of BAL fluid (12).

Human bronchial ECs culture

Human bronchial epithelial (HBE) cells were purchased from American Type Culture Collection (CRL-2741) and were cultured in RPMI 1640 (C11875500BT; Life Technologies) with 10% FBS (10082147; Life Technologies) and 1% penicillin-streptomycin (Beyotime Biotechnology) in 5% CO_2 at 37˚C (13). After human IL-33 (novoprotein, 500 ng/ml) stimulation for 15 min, 30 min, or 1 h; OVA (500 μm/l) stimulation for an hour; or LPS (100 μg/ml) for 24 h, cells were then subjected to further analysis. After Mito-TEMPO (5 μg/ml; Sigma-Aldrich) stimulation for half an hour followed by intervention of IL-33 for another half an hour, cells were then subjected to further analysis.

Small interfering RNA preparation and transfection

The cGAS small interfering RNA (siRNA; Santa Cruz Biotechnology) transfection was performed with the transfection reagent (GenMute siRNA Transfection Reagent; SignaGen Laboratories) following the manufacturer's protocol. Briefly, 5×10^4 cells were placed in each well of six-well plates for 18 h prior to transfection. The siRNA in 1 ml of growth medium were incubated with HBE cells for 5 h. The transfection medium was removed and replaced with 2 ml of fresh growth medium. Cells are stimulated by IL-33/OVA after transfection for 24 h.

Immunofluorescence analysis of dsDNA and cGAS levels in HBE cells and lung tissues of asthmatic mice

Sections of lung tissues of mice were immunostained with dsDNA Ab (ab27156; Abcam), CC10 Ab (ab40873; Abcam), or cGAS Ab (26416-1-AP; Proteintech) according to the manufacturer's instructions. HBE cells were fixed in 4% formaldehyde solution for 20 min and washed three times with PBS, then permeabilized with PBS containing 0.1% Triton X-100 (LC262801; Sigma-Aldrich) for 5 min, and blocked with Ab diluents of 5% BSA (B2064; Sigma-Aldrich) in PBS for 30 min. Subsequently, the cells were exposed to the cGAS Ab and/or the dsDNA Ab overnight at 4˚C, then washed briefly three times with PBS containing 0.1% Tween (Sigma-Aldrich) and stained with secondary Ab (Abcam) diluted in PBS containing 5% BSA for 1 h at room temperature. Finally, the cells mounted on slides in a drop of Vectashield plus DAPI mounting medium (Thermo Fisher Scientific) and/or the f-actin Ab (Phalloidin-iFluor 488; Abcam). Fluorescent images were captured with a Zeiss LSM laser scanning confocal microscope. Immunofluorescence-stained cells in airway epithelium of mice were measured double blind by the rate of cytosolic dsDNA–positive cells. The sum of the positive rate from each lung was divided by the number of airways examined (20–30 per mouse). The abundance of cytosolic dsDNA or cytosolic dsDNA colocalized with cGAS Ab in immunofluorescence-stained HBE cells was quantified in a double-blind manner by the number of positive dots per cell in high-power fields (10–20 cells per experiment).

Histopathologic evaluation of lung tissue

Lungs were fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with H&E/periodic acid–Schiff (PAS) reagent and observed under a microscope. All slides were examined in a random blind fashion by two independent investigators.

RNA isolation and real-time quantitative PCR

RNA was isolated from homogenized mouse lung and HBE cells using RNAiso Plus (Takara Biotechnology, Shiga, Japan). Reverse transcription was performed with Reverse Transcription Reagents (DRR037A; Takara Biotechnology). The expression of human GM-CSF and cGAS and mouse IL-4, IL-13, IL-25, IL-33, GM-CSF, and cGAS was measured by real-time quantitative PCR using SYBR Green Master Mix (DRR041A; Takara Biotechnology) on a StepOne real-time PCR system (Applied Biosystems, Foster City, CA). All protocols were performed according to the

manufacturer's instructions. The primers used for PCR experiments are listed in Supplemental Table I.

ELISA

BAL fluid and cell culture medium samples were centrifuged at 1000 rpm for 10 min, and the supernatants were collected and stored at −80°C. Inflammatory protein IL-4, IL-5, or IL-13 in the supernatants of cell medium or homogenate of lung tissue was measured by ELISA (Invitrogen), according to the manufacturer's instructions. BALF protein concentrations were determined by bicinchoninic acid protein assay kit (23227; Thermo Fisher Scientific).

Statistical analysis

Results are presented as mean ± SEMs. Data were analyzed with the Student *t* test (one tailed) or one-way ANOVA, followed by the Tukey post hoc test with GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Differences were considered statistically significant at a p value <0.05. Data are expressed as mean ± SEMs of individual groups from three independent experiments.

Results

Increased accumulation of cytosolic dsDNA in airway ECs in the setting of allergic inflammation, both in vitro and in vivo

A previous study has shown that HDM induces double-strand breaks in bronchial epithelium (5). It is well known that DNA damage leads to the accumulation of cytosolic DNA (14), so we investigated whether cytosolic dsDNA was increased in airway ECs in the setting of allergic inflammation. We first assessed the dsDNA accumulation in the airway ECs of asthmatic mouse models. OVA- or HDM-induced asthmatic mice model was established according to schemes in Fig. 1A–D. As expected, cytosolic dsDNA was increased in airway ECs of mice 3, 6 (reached peak value), and 12 h after the first challenge with an aerosol of 1% OVA (Fig. 1E) but not 24 h after the last challenge (data not shown). Similarly, the accumulation of cytosolic dsDNA appeared in airway ECs of mice only at 24 h (Fig. 1F), not at 72 h after last HDM challenge. The in vitro stimulation of HBE cells with IL-33 is usually used to mimic the allergic airway inflammation in vivo. Staining with an Ab against dsDNA revealed that the accumulation of cytosolic dsDNA was significantly increased in HBE cells treated with 500 ng of IL-33 stimulation for 15 min and 0.5 h but not for 1 h (Fig. 2). The results of dsDNA staining with secondary Ab alone turned out to be negative, which excluded false-positive results of cytosolic dsDNA staining (Supplemental Figs. 2, 3). These results implicated that the allergen- or IL-33–induced accumulation of cytosolic dsDNA in airway ECs was transient and only appeared in the early time of challenge.

Deletion of cGAS in the airway ECs significantly attenuated OVA-induced airway inflammation

cGAS is a DNA sensor that triggers innate immune response, so we investigated whether cGAS played a crucial role in asthma pathogenesis through sensing cytosolic dsDNA. Deletion of cGAS in the airway ECs was achieved by crossing floxed cGAS mice ($cGAS^{flox/flox}$) with CC10-rtTA/(tetO)7-Cre mice (Fig. 3A). As expected, deletion of cGAS in the airway ECs of OVA-challenged mice significantly reduced the total cell (Fig. 3B) and eosinophil cell (Fig. 3C, 3D) counts in BAL fluid and the IL-4, IL-5, and IL-13 mRNA (Fig. 3E–G) as well as protein levels (Fig. 3H–J) in lung homogenates. H&E staining showed that the inflammatory cell infiltration of lung tissues in $cGAS^{\triangle/\triangle}$ mice was markedly decreased compared with that in allergic wild-type (WT) mice, and the average H&E score was reduced from 2.6 of WT mice to 1.6 of $cGAS^{\triangle/\triangle}$ mice ($p < 0.05$, Fig. 3K).

Having determined the effects of cGAS deletion in OVA-induced airway inflammation, we then assessed whether it has the same efficacy in airway mucus hypersecretion and airway AHR. As expected, the extensive goblet cell hyperplasia and mucus production in OVA-challenged $cGAS^{\triangle/\triangle}$ mice were reduced compared with that in WT mice, and the average PAS score was decreased from 1.4 of WT mice to 0.4 of $cGAS^{\triangle/\triangle}$ mice ($p < 0.05$, Fig. 3L). Additionally, the airway AHR of $cGAS^{\triangle/\triangle}$ mice was also significantly reduced compared with that of WT mice (Fig. 3M).

Deletion of cGAS in the airway ECs markedly attenuated HDM-induced airway inflammation

HDM is a common airway allergen and plays an important role in initiating as well as sustaining inflammation through induction of T_H2 responses (15). To further explore the role of cGAS in asthma pathogenesis, we investigated whether cGAS deletion of airway ECs could also affect HDM-induced allergic airway inflammation. Deletion of cGAS in the airway ECs was achieved (Fig. 4A) by using the method described above. As expected, the total cell (Fig. 4B) and eosinophil cell (Fig. 4C, 4D) counts in BAL fluid and the IL-4 and IL-13 mRNA (Fig. 4E, 4F) as well as protein levels (Fig. 4G, 4H) in lung homogenates were markedly reduced in HDM-challenged $cGAS^{\triangle/\triangle}$ mice compared with those in allergic WT mice. Additionally, cGAS deletion in airway ECs of HDM-challenged mice also significantly inhibited the inflammatory cell infiltration and goblet cell hyperplasia in lung tissues (Fig. 4I, 4J).

cGAS deletion in airway ECs reduced GM-CSF, IL-25, and IL-33 production in the setting of allergic airway inflammation

We have shown strong evidence that cGAS deletion of airway ECs could markedly attenuate both OVA- and HDM-induced allergic airway inflammation. However, the detailed molecular mechanisms underlying how airway epithelial cGAS promotes T_H2 immune response remained to be elucidated. Many studies have confirmed that cytokines secreted by ECs such as IL-25, IL-33, and GM-CSF play a crucial role in cross-talk between airway ECs and T_H2 immune signaling, so the effects of cGAS deletion on production of these cytokines were explored. To exclude the influence of eosinophilic airway inflammation on production of IL-25, IL-33, and GM-CSF, we used a short time point asthmatic model as described above (Fig. 1B). Unexpectedly, at 3 h after the first challenge with OVA (the time point at which eosinophils in BALF, IL-4, IL-5, and IL-13 in lung homogenates have not begun to increase [data not shown]), only the expression of GM-CSF (Fig. 5A) not IL-25 and IL-33 mRNA (Fig. 5B, 5C) was significantly decreased in OVA-induced $cGAS^{\triangle/\triangle}$ mice compared with those of control mice. These data suggested cGAS participated in initiating OVA-induced allergic airway inflammation likely via regulating GM-CSF production in early stage. Additionally, in conventional asthmatic model (Fig. 1A, 1C), cGAS deletion markedly reduced the expression of IL-25 and IL-33 not GM-CSF in lung homogenates of OVA- or HDM-challenged mice (Fig. 5D–J). However, the inhibition of IL-25 and IL-33 production was likely due to the attenuation of eosinophilic airway inflammation by cGAS in conventional asthmatic model.

Knockdown of cGAS reduced GM-CSF production in IL-33– or OVA-induced HBE cells

To further explore the mechanisms underlying how cGAS promotes T_H2 immunity, we investigated whether knockdown of cGAS could inhibit the production of IL-25, IL-33, and GM-CSF in HBE cells in response to IL-33 or OVA stimulation. Interestingly, consistent with in vivo results as described above, after knockdown of cGAS by siRNA (Fig. 6A), only the expression of GM-CSF mRNA, not IL-25 and IL-33 mRNA, was

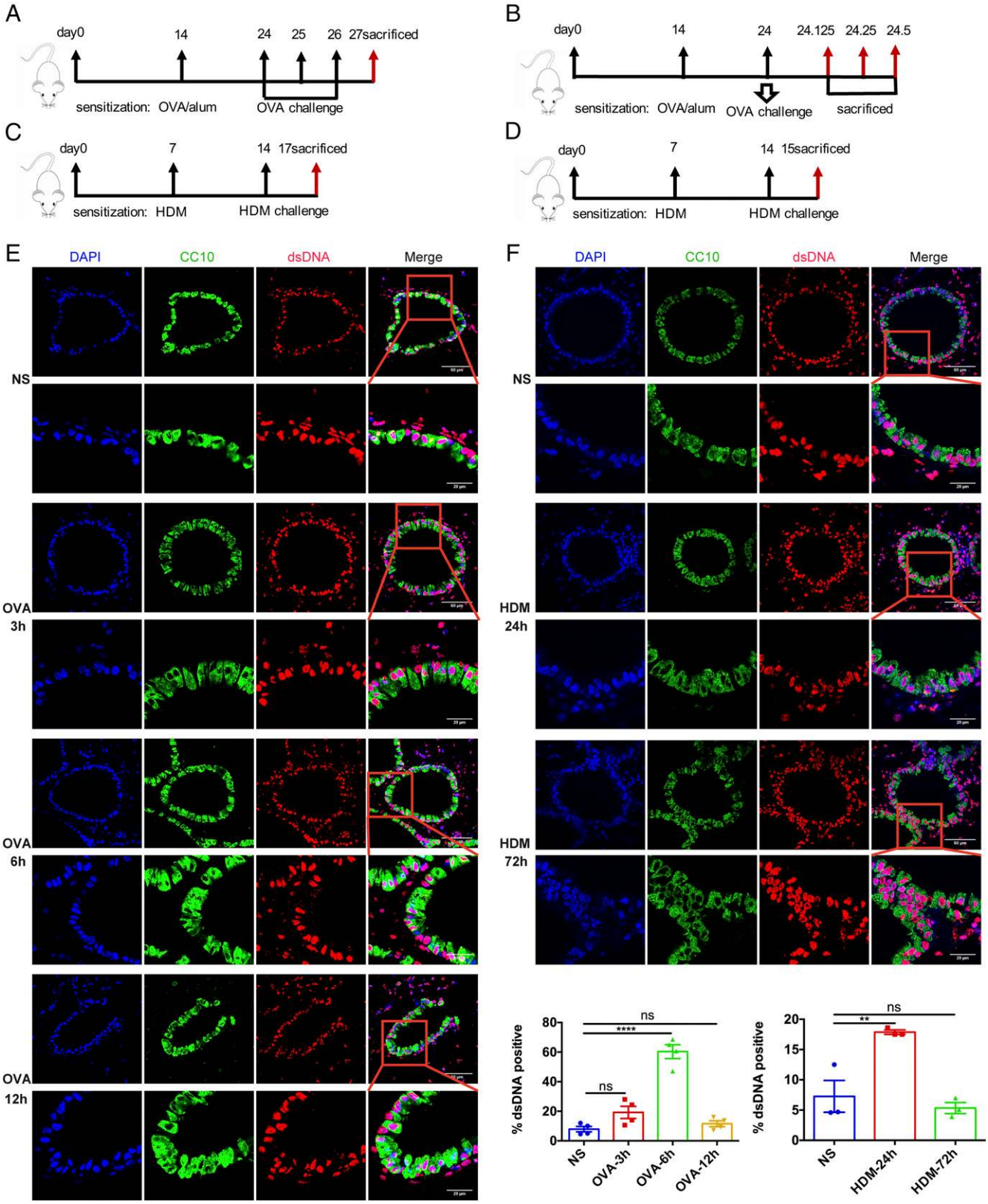

FIGURE 1. Increased accumulation of cytosolic dsDNA in airway ECs in the setting of allergic inflammation in vivo. (**A**–**D**) OVA- (conventional or short time point) or HDM-challenged asthmatic mouse models shown in (A)–(D). (**E**) dsDNA accumulation in the airway ECs of OVA/alum-induced asthma in vivo model shown in (B) at 3, 6, and 12 h after the last OVA challenge was shown by immunofluorescence assays. (**F**) dsDNA accumulation in the airway ECs of HDM-induced asthma in vivo model shown in (C) and (D) at 24 and 72 h, respectively, was shown by immunofluorescence assays. Scale bars, 60 μm (main images) and 20 μm (enlargements), respectively. The quantitative results of dsDNA positive cell percentage were shown in (F). Data are the mean ± SEM of two independent experiments, with 20–30 airways per mouse counted with a total of $n = 3$–4 mice per group. $^{**}p < 0.01$, $^{****}p < 0.0001$.

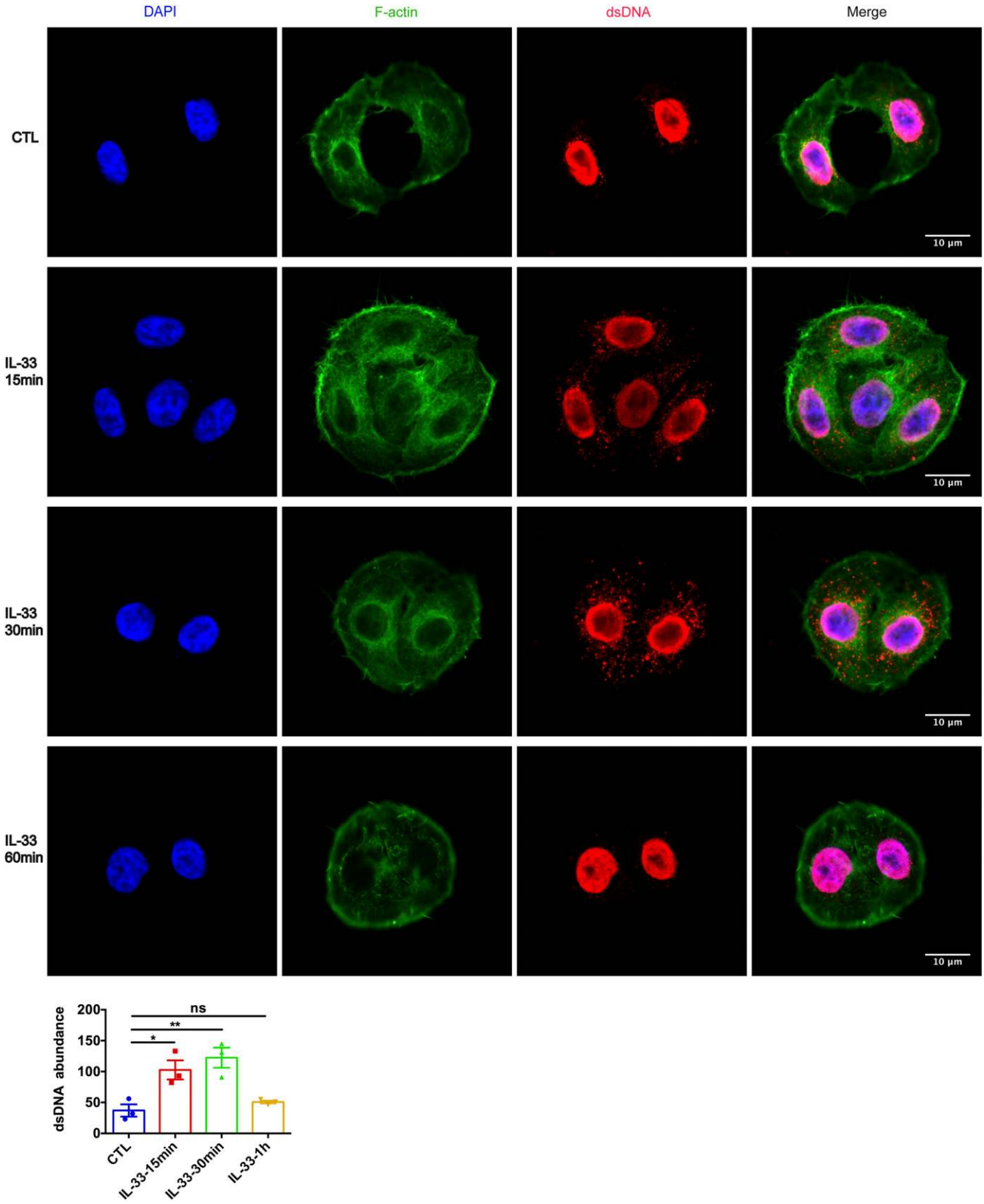

FIGURE 2. Cytosolic dsDNA augmentation in HBE cells with IL-33 treatment in vitro. dsDNA accumulation in the HBE cells at 15, 30, and 60 min when treated with 500 ng of IL-33 was shown by immunofluorescence assays. The quantitative results of cytosolic dsDNA abundance in HBE cells were shown. Data are the mean ± SEM of three independent experiments, with 10–20 cells per experiment. $*p < 0.05$, $**p < 0.01$.

significantly inhibited in HBE cells treated with 500 ng of IL-33 for 0.5 h (Fig. 6B). Additionally, we also found that knockdown of cGAS largely abrogated the GM-CSF mRNA and protein production in HBE cells stimulated with 500 μM OVA for 1 h (Fig. 6C, 6D). To exclude the interference of trace quantity of LPS mixed in OVA, HBE cells were stimulated with different concentrations of LPS. We found GM-CSF mRNA expression was markedly induced only by high dose of LPS

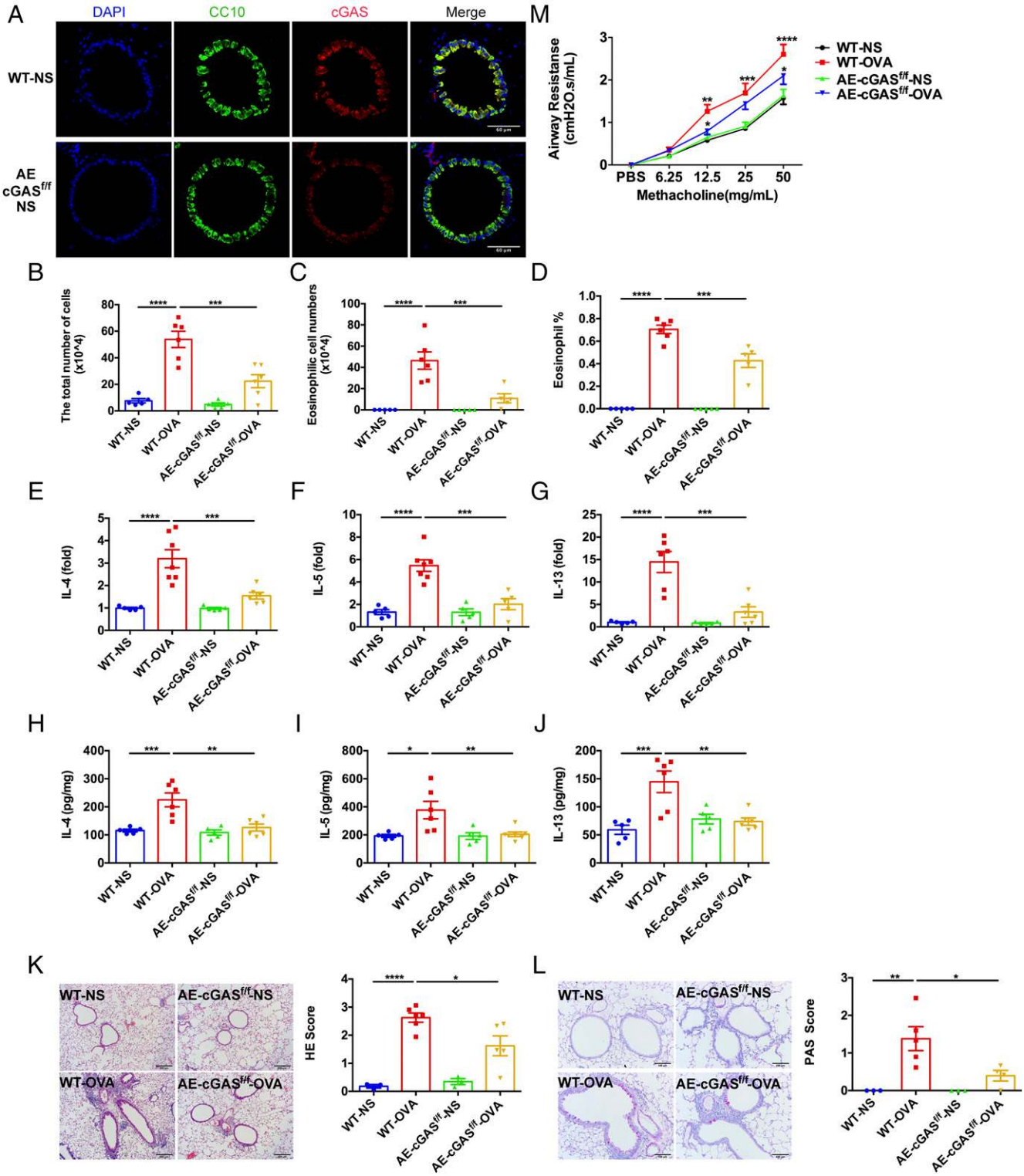

FIGURE 3. OVA-induced airway inflammation attenuated by deletion of cGAS in airway ECs. (**A**) cGAS expression in the airway ECs was verified by immunofluorescence assays. Scale bars, 60 μm. (**B**–**D**) Total BAL cells (B), eosinophil numbers (C), and percentages (D) were counted. (**E**–**G**) Relative mRNA levels of IL-4 (E), IL-5 (F), and IL-13 (G) were measured by quantitative RT-PCR. (**H**–**J**) IL-4 (H), IL-5 (I), and IL-13 (J) levels in lung homogenates. (**K** and **L**) H&E staining (K) and PAS staining (L) were used to identify inflammatory cell recruitment. H&E (K) as well as PAS score (L) were counted. Scale bars, 200 μm (K) and 100 μm (L). (**M**) AHR data. Data are the mean ± SEM of two independent experiments with a total of $n = 5$–7 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(Fig. 6E). Furthermore, we explored whether cGAS deletion could also reduce the GM-CSF expression in HBE cells treated with LPS. Contrary to the situation of OVA challenge, knockdown of cGAS markedly promoted the production of GM-CSF mRNA in HBE cells treated with 100 μg of LPS for 24 h (Fig. 6F). These data provided strong evidence that the effects of OVA challenge on cGAS$^{-/-}$ HBE cells solely relied on OVA itself, not the effects of LPS. These in vitro results provided

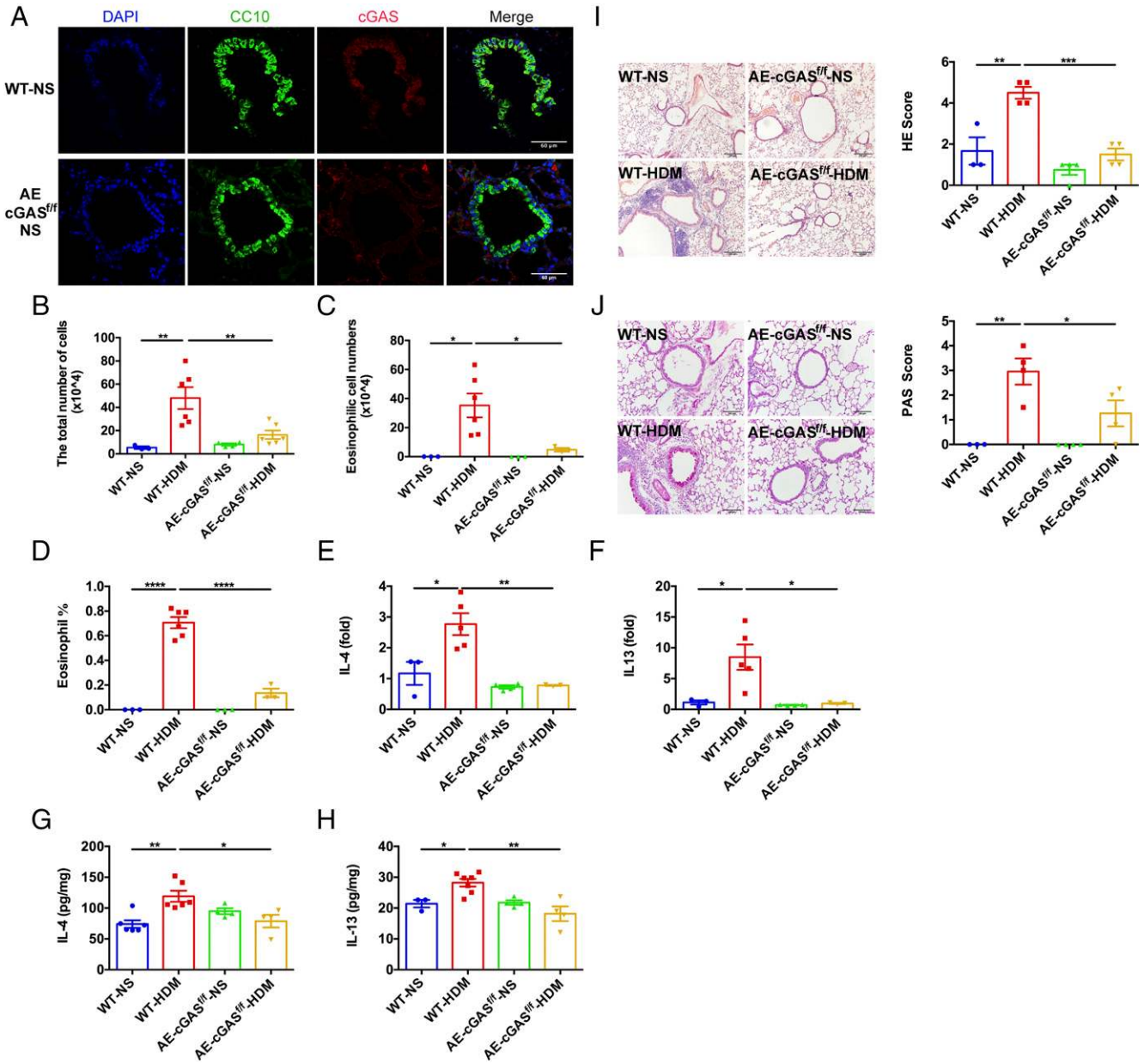

FIGURE 4. HDM-induced airway inflammation attenuated by deletion of cGAS in airway ECs. (**A**) cGAS expression in the airway ECs was verified by immunofluorescence assays. Scale bars, 60 μm. (**B**–**D**) Total BAL cells (B), eosinophil numbers (C), and percentages (D) were counted. (**E** and **F**) Relative mRNA levels of IL-4 (E) and IL-13 (F) were measured by quantitative RT-PCR. (**G** and **H**) IL-4 (G), and IL-13 (H) levels in lung homogenates were measured by ELISA. (**I** and **J**) H&E staining (I) and PAS staining (J) were used to identify inflammatory cell recruitment. H&E (I) as well as PAS score (J) were counted. Scale bars, 200 μm (I) and 100 μm (J). Data are the mean ± SEM of two independent experiments with a total of $n = 3$–6 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

further evidence that cGAS promoted T_H2 immunity likely via regulating GM-CSF production.

Cytosolic dsDNA accumulation was partially inhibited by Mito-TEMPO and colocalized with cGAS in HBE cells treated with IL-33

We have demonstrated that IL-33 markedly upregulated cytosolic dsDNA production in HBE cells. However, the mechanisms underlying this process remain unclear. Previous studies confirmed mitochondria damage could lead to the release of mtDNA fragments into cytosol (9), and the mitochondria-targeted antioxidant Mito-TEMPO, a scavenger specific for mitochondrial reactive oxygen species, could suppress the release of mtDNA into the cytosol triggered by treatment with LPS via decreasing mitochondrial membrane permeability (16). In this study, we investigated whether Mito-TEMPO could reduce IL-33–induced cytoplasmic dsDNA production, possibly through suppressing the release of mtDNA into the cytosol. As expected, after pretreatment with Mito-TEMPO, the percentage of cytosolic dsDNA–positive HBE cells stimulated with IL-33 was significantly decreased (Fig. 7A). Furthermore, Mito-TEMPO also decreased the IL-33–induced GM-CSF mRNA expression in HBE cells (Fig. 7B). To provide further evidence of dsDNA sensed by cGAS in IL-33–induced HBE cells, we used confocal microscopy images of HBE cells stained with anti-dsDNA and anti-cGAS Abs. As shown in Fig. 7C, the percentage of dsDNA colocalization with cGAS was markedly increased in IL-33–induced HBE cells compared with that in control cells.

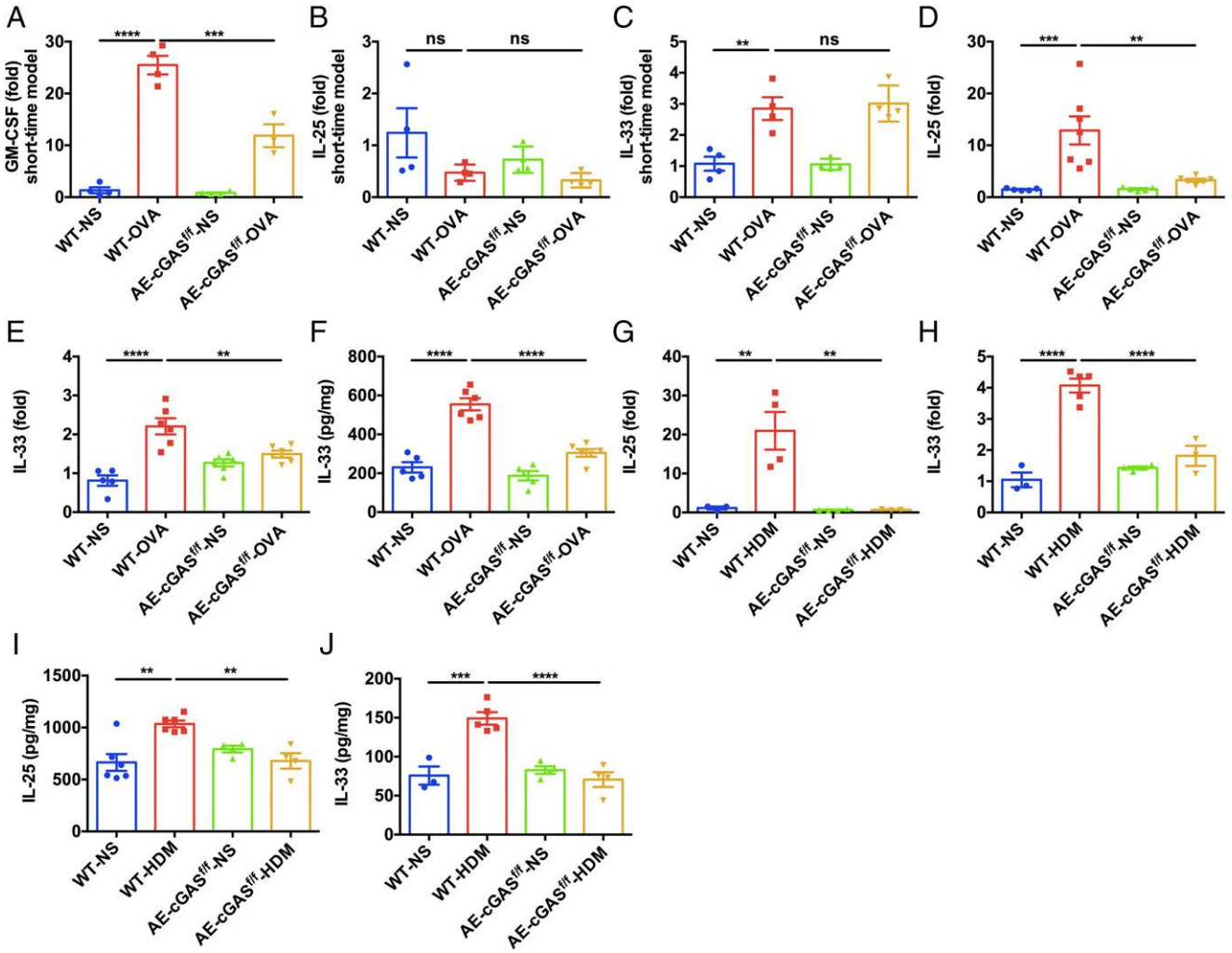

FIGURE 5. cGAS deletion of airway ECs reduced GM-CSF, IL-25, and IL-33 production in OVA- or HDM-challenged asthmatic mice. (**A**–**C**) GM-CSF (A), IL-25 (B), and IL-33 (C) mRNA levels in lung homogenates of OVA-challenged (short time point) mice were assessed by using real-time quantitative PCR (RT-qPCR). (**D**–**F**) IL-25 mRNA levels (D), IL-33 mRNA levels (E), and IL-33 protein levels (F) in lung homogenates of OVA-challenged mice were assessed by using RT-qPCR and ELISA. (**G**–**J**) IL-25 mRNA levels (G), IL-33 mRNA (H), IL-25 protein levels (I) and IL-33 protein levels (J) in lung homogenates of HDM-challenged mice were assessed by using RT-qPCR and ELISA. Data are the mean ± SEM of two independent experiments with a total of n = 3–7 mice per group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Discussion

In this study, to our knowledge, we demonstrated for the first time that the accumulation of cytosolic dsDNA from airway ECs was markedly increased in the setting of allergic inflammation, and cGAS deletion in airway ECs significantly attenuated OVA- or HDM-induced T_H2 immune response in mice. Furthermore, we found cGAS promoted T_H2 allergic inflammation likely via regulating airway epithelial GM-CSF production and may play an important role in innate as well as adaptive immune responses of asthma pathophysiology.

In innate immune cells, activation of the cGAS triggers the type I IFN response, which has evolved as a major protective immune defense mechanism for the detection and suppression of virus- or bacteria-induced infection (17–19). However, overactivation of cGAS followed by an overproduction of harmful proinflammatory cytokines has been found to play an important role in the pathogenesis of some autoimmune disease (20–22), acute pancreatitis (23), and insulin resistance (10). Our study showed that inhibition of cGAS reduced allergic airway inflammation and AHR and suggested that cGAS had the potential to be a new target for asthma therapy. Additionally, there are some studies that explore the mechanisms by which airway ECs influence immune cells in asthma pathogenesis. Causton et al. (24) demonstrated that genetically modified mice with CARMA3 (specifically expressed in airway ECs and mediates NF-κB activation in these cells in response to stimulation)-deficient airway ECs have reduced airway eosinophilia and proinflammatory cytokine production in a murine model of allergic airway inflammation. Another study showed airway EC-derived CSF1 had a critical role in the recruitment of alveolar DCs in asthmatic mice, and this study linked the innate and adaptive immune responses in T_H2 cell–mediated allergic lung inflammation (25). Therefore, our study further clarified the molecular mechanisms by which airway ECs in response to various inhaled allergens influenced the innate immune response in asthma.

We demonstrated that cGAS deletion of airway ECs could markedly inhibit production of GM-CSF in OVA-challenged (short time point) asthmatic mice. However, how cGAS promotes T_H2 immunity via GM-CSF still lacks direct evidence. It is of interest to note that, although the fold induction of GM-CSF induced by OVA was similar in each siRNA-treated cell, the baseline and

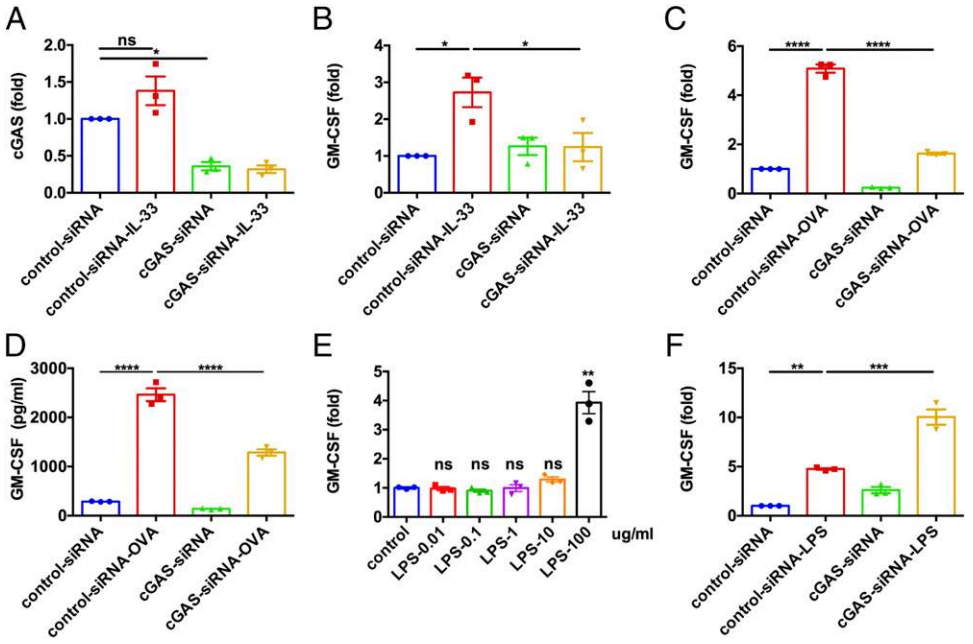

FIGURE 6. Knockdown of cGAS reduced GM-CSF production in IL-33– or OVA-induced HBE cells. (**A** and **B**) After treatment with IL-33, relative mRNA levels of cGAS (A) and GM-CSF (B) were measured by real-time quantitative PCR (RT-qPCR) in cGAS$^{-/-}$ HBE cells and controls. (**C** and **D**) After treatment with OVA, relative mRNA (C) and protein (D) levels of GM-CSF were measured by using RT-qPCR and ELISA in cGAS$^{-/-}$ HBE cells and controls. (**E**) After treatment with different dose of LPS, relative mRNA levels of GM-CSF were measured by RT-qPCR in HBE cells. (**F**) After treatment with LPS, relative mRNA levels of GM-CSF were measured by RT-qPCR in cGAS$^{-/-}$ HBE cells and controls. Data are the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

eventually the OVA-induced levels of GM-CSF expression were decreased when cGAS was inhibited, suggesting that cGAS plays an essential role in regulation of basal GM-CSF production in airways. In agreement with this, Hwang et al. (26) have also observed similar findings in fibronectin fragment-induced GM-CSF expression in chondrocytes. In contrast, the innate cytokine GM-CSF has the propensity to activate DCs that prime T_H2 responses (27, 28). Therefore, whether cGAS deletion of airway ECs could influence the activation of DCs in asthmatic mice is required to be further explored.

A recent study (11) reported rhinovirus infection triggers dsDNA release associated with NETosis in a mouse model of asthma, and degrading dsDNA with DNase protects mice from T_H2 immunopathology. However, this study did not detect cytosolic dsDNA of airway EC and also did not further explore the mechanisms by which dsDNA from neutrophil extracellular traps triggers T_H2 immune response. Whether cGAS also plays an important role in asthma exacerbation induced by dsDNA derived from NETosis needs to be further investigated. Additionally, TLR4 as one of the most common PRRs has been demonstrated to mediate allergic asthma (29), but these effects were only limited to HDM, not generalized to other allergens. For example, Shalaby et al. (30) showed that inhaled birch pollen extract–induced airway AHR was not dependent on TLR4. Whether cGAS mediates various allergen-induced allergic airway inflammation is worth further exploration.

Interestingly, we found that the upregulation of cytosolic dsDNA was transient in airway ECs both in vitro and in vivo. Normally when nuclear or mtDNA damage followed by accumulation of cytosolic DNA is present, cells themselves have a strong ability to eliminate cytosolic DNA to avoid triggering innate inflammation. For example, TREX1 as an exonuclease can degrade cytosolic DNA (31–34), and Trex1 deficiency in humans has been linked to several autoimmune and inflammatory diseases because of the upregulation of cytosolic dsDNA (35). Therefore, we speculated that the accumulation of cytosolic dsDNA was only detected in the early time of OVA or HDM challenge just because the repair effects of cells have not completely developed. Furthermore, consistent with dsDNA accumulation, our study found that cGAS deletion abrogated GM-CSF production only in the early time of OVA challenge. Collectively, our data indicated that the accumulation of cytosolic dsDNA in airway ECs may be mainly involved in the asthma pathogenesis just limited in the early stage of allergen challenge.

mtDNA is prone to damage, owing to its lack of packaging by histones and inefficient DNA repair mechanisms. Many previous studies showed that mitochondria damage is associated with asthma pathophysiology. Boldogh et al. (36) showed that mitochondrial dysfunction induced by oxidant environmental pollutants is responsible for the severe symptoms in allergic airway inflammation. Ghosh et al. (37) reported that mitochondrial structural changes and dysfunction are associated with allergic asthma. Anderson et al. (38) confirmed that mitochondrial-targeted antioxidant therapy decreases TGF-β–mediated collagen production in a murine asthma model. However, these studies did not involve mtDNA damage and subsequent accumulation of cytosolic DNA. Although most endogenous ligands for cGAS are presumed to originate from nuclear DNA, several lines of evidence now suggest that mtDNA also serves as a cell-intrinsic cGAS ligand in certain contexts (39–41). In fact, a recent study confirmed mtDNA stress could lead to escape of mtDNA into the cytosol and prime the antiviral innate immune response via activating the GAS–STING pathway (9). Similarly, we found that the mitochondria-targeted antioxidant Mito-TEMPO could reduce the IL-33–induced accumulation of cytosolic dsDNA and GM-CSF production in HBE cells, suggested the mtDNA released into cytosol may be one of main resources of cytosolic dsDNA, and could be sensed by cGAS to mediate asthma pathogenesis. However, whether the mtDNA was damaged and released into

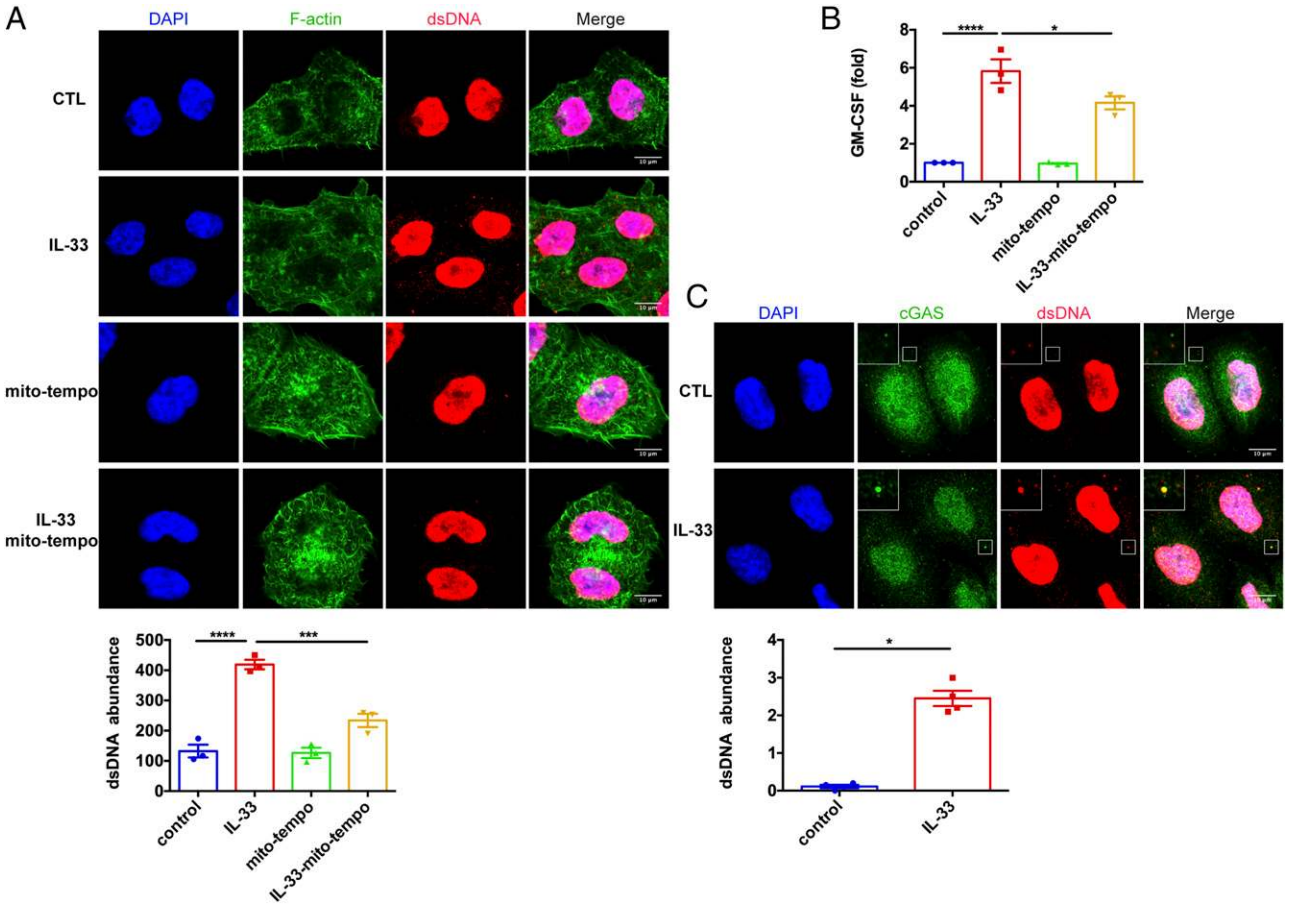

FIGURE 7. Cytosolic dsDNA accumulation was partially inhibited by Mito-TEMPO and colocalized with cGAS in IL-33–treated HBE cells. (**A**) Representative images of immunofluorescence staining for dsDNA in HBE cells treated with IL-33 and Mito-TEMPO in vitro. The quantitative results of cytosolic dsDNA abundance of three independent experiments in HBE cells were shown (10–20 cells per experiment). (**B**) Relative mRNA levels of GM-CSF in HBE cells treated with IL-33 and Mito-TEMPO in vitro. Data are the mean ± SEM of three independent experiments. (**C**) Representative images of immunofluorescence staining for dsDNA (staining red) and cGAS (staining green) in HBE cells treated with IL-33 in vitro. The quantitative results of cytosolic dsDNA colocalized with cGAS in HBE cells were shown. Data are mean ± SEM of four independent experiments, with 10–20 cells per experiment. Scale bars, 10 μm. $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$.

cytosol still lacked direct evidence in our study and needed to be further investigated.

It is well known that cGAS is dependent on STING to activate TBK1, NF-κB, and IRF3 to promote the production of type I IFNs and other cytokines (28, 42). Lei et al. (43) showed cGAS-mediated autophagy protects the liver from ischemia-reperfusion injury independently of STING, and Liu et al. (44) confirmed only THP1-derived macrophages but not airway-derived cells showed significant response to immunostimulatory DNA, HSV-60mer dsDNA, and VACV-70mer dsDNA, suggesting that the airway ECs have a defect in the STING-dependent sensing pathway. Therefore, whether airway epithelial STING also has an important role in airway EC-mediated asthma pathogenesis was required to be further investigated.

In conclusion, we confirmed airway epithelial cGAS played an important role in asthma pathogenesis and was a promising therapeutic target for allergic airway inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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